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(54) Method for determining gene essentiality in a pathogen

(57) The present invention provides a method for determining pathogen sensitivity to varying levels of reduction of a gene product using an expression vector system having a promoter that is essentially off *in vitro* and turns on selectively during the infection process *in vivo*. Genes and gene products identified by this method as essential to growth of infection of a selected pathogen are also provided. In addition, therapeutic compositions designed to target genes identified by this method are provided.

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Description

RELATED APPLICATIONS

This Application claims benefit of U.S. Provisional Applications Serial Number 60/028,416, filed October 15, 1996, and Serial Number 60/031,161, filed November 18, 1996.

FIELD OF THE INVENTION

The present invention provides a method for determining pathogen sensitivity to varying levels of reduction of expression of a gene product using an expression vector system having a promoter that is essentially "off' in vitro and turns "on" selectively during the infection process. Using this method, gene targets most sensitive to inhibition can be selected as molecular targets for the development of new therapies against selected pathogens.

BACKGROUND OF THE INVENTION

Identification, sequencing and characterization of genes is a major goal of modern scientific research. By identifying genes, determining their sequences and characterizing their biological function, it is possible to employ recombinant technology to produce large quantities of valuable gene products, e.g. proteins and peptides. Additionally, knowledge of gene sequences can provide a key to diagnosis, prognosis and treatment in a variety of disease states in plants and animals which are characterized by inappropriate expression and/or repression of selected genes or by the influence of external factors, e.g., carcinogens or teratogens, on gene function.

A variety of techniques have also been described for identifying particular gene sequences on the basis of their gene products. For example, see International Patent Application No. WO91/07087, published May 30, 1991. In addition, methods have been described for the amplification of desired sequences. For example, see International Patent Application No. WO91/17271, published November 14, 1991.

Genes which are essential for the growth of an organism, however, have been difficult to identify in such a manner as to be easily recovered for future analysis. The most common methodology currently employed to identify essential genes is a multi-step process involving the generation of a conditionally lethal mutant library followed by the screening of duplicate members under the appropriate permissive and non-permissive conditions. Candidate mutants are then transformed with a second, genomic library and the desired genes isolated by complementation of the mutant phenotype. The complementing plasmid is recovered, subcloned, and then retested. However, this procedure comprises multiple subcloning steps to identify and recover the desired genes thus making it both labor intensive and time consuming.

A number of approaches for the isolation of pathogen virulence genes based upon transposon mutagenesis have been developed. These include screening for the loss of specific virulence-associated factors (Lee et al. *J. Infect. Dis.* 1987, *156*:741), survival within macrophages (Fields et al. *Proc. Nat'l Acad. Sci.* 1986, *83*:5189), and penetration of epithelial cells (Finlay et al. *Mol. Microbiol.* 1988, *2*:757). However, these methods are restricted to certain stages of infection.

Transposon mutants have also been tested in live animal models of infection (Miller et al. *Infect. Immun.*, 1989, *57*:2758; and Bolker et al., *Mol. Gen. Genet.*, 1994, *248*:547-552). However, comprehensive screening of bacterial genes is not possible due to the inability to identify mutants with attenuated virulence within pools of mutagenized bacteria and thus the huge number of mutants would require individual screening.

Hensel et al. have developed an insertional mutagenesis system that uses transposons carrying unique DNA sequence tags for the isolation of bacterial virulence genes. *Science,* 1995, *269*:400-403. In this system, termed signature-tagged mutagenesis, each transposon mutant is tagged with a different DNA sequence. This permits identification of bacteria recovered from hosts infected with a mixed population of mutants, as well as the selection of mutants with attenuated virulence. This method was used to identify virulence genes of *Salmonella typhimurium* in a murine model of typhoid fever. Further, Slauch et al. describe a method referred to as IVET technology which provides a means for identifying transcripts which are essentially absent *in vitro,* but are on throughout, or during, various phases of infection *(Methods in Enzymology* 1994, *235*:481-492). However, these methods only provide information on the effect of the total absence or the specific up-regulation *in vivo* of the gene product in the organism.

Accordingly, there exists an unmet need for an efficient method of identifying varying levels of genes essential to the infectivity and growth of a pathogen.

SUMMARY OF THE INVENTION

In one aspect, the invention provides a method of identifying varying levels of a gene or genes that are essential

to the infectivity and growth of a pathogen through the use of an expression vector system which uses a promoter that is "off' *in vitro,* and turns "on" selectively during a particular phase of the infection process of a pathogen.

An additional aspect of the invention provides an isolated gene which is essential to the infectivity and/or growth of a pathogen and is identified by the above method.

Yet another aspect of the invention is an isolated protein produced by expression of the gene sequence identified above. Such proteins are useful in the development of therapeutic and diagnostic compositions, or as targets for drug development.

Yet another aspect of the invention is to identify antibiotics, especially broad spectrum antibiotics, and particularly those that modulate the expression of these essential genes and/or activity of their gene products.

The invention provides a method of determining gene essentiality in a selected pathogen comprising: preparing a vector expression system comprising a promoter of the potential target gene having on/off characteristics and a reporter gene; expressibly linking the target gene promoter in the vector expression system with a ribozyme or antisense construct for the potential target gene; introducing the expression vector system containing the ribozyme or antisense construct into a selected pathogen; infecting at least one animal with the pathogen containing the expression vector system; determining gene expression levels of the target gene; and correlating target gene expression levels with progression of the infection in the animal to identify genes essential to growth of the selected pathogen.

The invention also provides method of determining gene essentiality in a selected pathogen comprising: preparing a vector expression system comprising a promoter of the potential target gene having on/off characteristics and a reporter gene; expressibly linking the target gene promoter in the vector expression system with a ribozyme or antisense construct for the potential target gene; introducing the expression vector system containing the ribozyme or antisense construct into a selected pathogen; infecting at least one animal with the pathogen containing the expression vector system; determining levels of mRNA of the target gene; and correlating levels of mRNA with progression of the infection in the animal to identify genes essential to growth of the selected pathogen.

Still further provided by the invention is a method of determining gene essentiality in a selected pathogen comprising: identifying a potential target gene; preparing a vector expression system comprising a promoter of the potential target gene having on/off characteristics and a reporter gene; confirming that the on/off characteristics of the promoter in the vector expression system are preserved; replacing the reporter gene in the vector expression system with a ribozyme or antisense construct for the potential target gene; introducing the expression vector system containing the ribozyme or antisense construct into a selected pathogen; infecting at least one animal with the pathogen containing the expression vector system; determining levels of mRNA of the target gene; and correlating levels of mRNA with progression of the infection in the animal to identify genes essential to growth of the selected pathogen.

A method is also provided of determining gene essentiality in a selected pathogen comprising: preparing a vector expression system comprising a promoter of the potential target gene having on/off characteristics and a reporter gene; expressibly linking the target gene promoter in the vector expression system with a ribozyme or antisense construct for the potential target gene; introducing the expression vector system containing the ribozyme or antisense construct into a selected pathogen; infecting at least one animal with the pathogen containing the expression vector system; determining levels of protein of expressed by the target gene; and correlating levels of protein with progression of the infection in the animal to identify genes and gene products essential to growth of the selected pathogen.

Methods are also provided wherein the selected pathogen is selected from the group consisting of *Streptococcus, Streptococcus pneumoniae, Staphylococcus, Staphylococcus aureus, Enterococcus, Enterococcus faecalis, Pseudomonas, Pseudomonas auriginosa, Escherichia,* and *Escherichia coli.*

Other methods are provided wherein the animal of the infecting step is selected from the group consisting of a rodent, a mouse, a rat, a rabbit, and a guinea pig.

The invention still further provides a kit comprising the vectors of the invention.

Provided by the invention are vector used in the methods of the invention, pathogen used in such methods.

Expression vectors comprising a promoter of a potential target gene having on/off characteristics and a reporter gene are also provided. Also provided are vectors comprising a target gene promoter expressibly linked to a ribozyme or antisense construct for the potential target gene.

The invention also provides a pathogen comprising an expression vector comprising a promoter of a potential target gene having on/off characteristics and a reporter gene, as well as a pathogen comprising a vector comprising a target gene promoter expressibly linked to a ribozyme or antisense construct for the potential target gene. Animals comprising such pathogens are also provided by the invention.

Other objects, features, advantages and aspects of the present invention will become apparent to those of skill in the art from the following description. It should be understood, however, that the following description and the specific examples, while indicating preferred embodiments of the invention, are given by way of illustration only. Various changes and modifications within the spirit and scope of the disclosed invention will become readily apparent to those skilled in the art from reading the following description and from reading the other parts of the present disclosure.

DETAILED DESCRIPTION OF THE INVENTION

The biochemical basis of many pathogen resistance mechanisms to antimicrobials is now known. These mechanisms alone, or in concert, are responsible for the escalating problem of antimicrobial resistance seen in both hospital and community acquired infections. The principle approach by researchers to overcome these problems has been to seek incremental improvements in existing drugs. While these approaches contribute somewhat to the fight against infection by such resistant pathogens, new approaches are needed.

Knowledge of genes or gene products essential to the growth of an organism can provide a key to the development of treatments of infectious pathogens. Gene knockout studies provide information on the effect of the total absence of a gene product. However, antimicrobial therapies can rarely achieve the complete abolition of activity of a given gene product. In addition, gene knockouts cannot be created (by simple insertion/deletion mutagenesis) if the gene products are essential to viability *in vitro*.

The present invention provides a method for determining pathogen sensitivity to varying levels of reduction of a gene product and is applicable to genes essential *in vitro* since reduction in levels of the gene product only occurs *in vivo*. The degree of mRNA reduction for a selected target can be monitored and correlated with the progression of the infection and/or viable counts recovered from infected tissue. Using this method, genes from a selected pathogen which are most sensitive to inhibition *in vivo* can be identified and selected as targets for the development of new intervention therapies.

By "pathogen" it is meant any organism which is capable of infecting an animal or plant and replicating its nucleic acid sequences in the cells or tissue of the animal or plant. Such a pathogen is generally associated with a disease condition in the infected animal or plant. Such pathogens may include, but are not limited to, viruses, which replicate intra- or extra-cellularly, or other organisms such as bacteria, fungi or parasites, which generally infect tissues or the blood. Certain pathogens are known to exist in sequential and distinguishable stages of development, e.g., latent stages, infective stages, and stages which cause symptomatic diseases. In these different states, the pathogen is anticipated to rely upon different genes as essential for survival.

Preferred pathogens useful in the methods of the invention include, for example, *Streptococcus, Streptococcus pneumoniae, Staphylococcus, Staphylococcus aureus, Enterococcus, Enterococcus faecalis, Pseudomonas, Pseudomonas auriginosa, Escherichia,* and *Escherichia coli.*

In this method, potential gene targets for antimicrobial therapy are first identified by either their essentiality for growth *in vitro* or their essentiality in infection. Essentiality for growth *in vitro* is determined for example by generation of conditional lethal mutants of a selected pathogen. The mutated gene can be identified by complementation with a genomic library prepared from the pathogen. Essentiality of a gene in the infection process is determined by generating individual gene deletions/knockouts or by using a random mutagenic approach followed by negative selection after cycling through an infection model such as signature tagged mutagenesis (STM; Hensel et al., *Science,* 1995, *269:* 400-403).

Precedents for the existence of genes whose transcription is essentially off *in vitro* but on in *vivo* have been highlighted by the IVET technology (Slauch et al., *Methods in Enzymology,* 1994, *235:*481-492) and RT-PCR analysis of total RNA isolated from infected tissue or *in vitro* grown cells. Using genome databases, primer pairs are designed to predicted transcripts of the selected pathogen and arrayed in microtiter dish format. Total RNA is isolated from an *in vitro* grown pathogen and RT-PCR performed with all the primer pairs. Similarly RT-PCR is performed with total RNA isolated at varying times from infections of the selected pathogen in a variety of appropriate animal models. Comparison of the PCR profiles which reflect the ratio of a given mRNA to internal standards such as rRNA or housekeeping genes provides identification of those transcripts which are essentially absent *in vitro,* but are on throughout, or during various phases, of infection.

Having identified potential target genes, the promoter region of the potential target gene is then cloned upstream of a reporter gene in a vector appropriate for the selected pathogen. By "appropriate" it is meant a vector capable of replicating stably in a selected pathogen. The present invention is based upon the use of expression vector systems which use a promoter that is essentially "off' *in vitro* and turns "on" selectively during a particular phase of the infection process. By "on" it is meant that the promoter functions in its normal capacity by inducing or promoting expression of the attached gene and that transcription is detectable; by "off' it is meant that the promoter does not function in its normal capacity thus resulting in very little or no detectable transcription. Preservation of the conditional and temporal expression of the promoter, or its "on"/"off" characteristics, within this new construct, during *in vitro* and *in vivo* growth, is confirmed visually (luminescence) and by RT-PCR using primers specific to the reporter mRNA. Upon confirmation of the preservation of these characteristics by the promoter, the reporter gene coding region is replaced with a ribozyme or antisense RNA construct for the potential target gene. Optimization of the ribozyme or antisense RNA to inhibit target gene expression is first carried out *in vitro* using a standard controllable promoter such as a T7 promoter coupled with IPTG/lacIPO controlled expression of T7 RNA polymerase.

The expression vector is then introduced into the selected pathogen using standard techniques. Introduction of

the vector carrying the ribozyme or antisense RNA construct into the selected pathogen should not affect growth or expression of the target gene *in vitro*. Introduction of the construct into animal models, however, results in the expression of the ribozyme or antisense RNA construct resulting in a reduction in target gene expression. Levels of gene expression can be monitored by RT-PCR of total mRNA isolated from infected tissue at various times during the infection and correlated with housekeeping gene controls and viable cell counts. Reduction in target mRNA is correlated with infection progression including disease pathology, luminescence in thin tissue sections to allow determination of the numbers of metabolically active pathogens and viable cell counts to prioritize gene targets for development of therapeutic agents. For example, in those cases where a significant reduction in target RNA, but little effect on viable cell count, is seen, the gene will be considered to be a less attractive target than situations where reduction in viable counts correlates with decreased target mRNA by RT-PCR analysis.

Genes and gene products identified according to the method of the present invention may then be used in the design of therapeutic and diagnostic agents. For example, genes identified in accordance with this method as essential to a selected pathogen in the infection process and proteins encoded thereby may serve as targets for the screening and development of natural or synthetic chemical compounds which have utility as therapeutic drugs for the treatment of infection by this pathogen. As an example, a compound capable of binding to such protein encoded by such gene and inhibiting its biological activity may be useful as a drug component preventing diseases or disorders resulting from the growth of a particular organism. Alternatively, compounds which inhibit expression or reduce expression of an essential gene are also believed to be useful therapeutically.

Conventional assays and techniques may be used for screening and development of such therapeutics. For example, a method for identifying compounds which specifically bind to or inhibit proteins encoded by these gene sequences can include simply the steps of contacting a selected protein or gene product with a test compound to permit binding of the test compound to the protein; and determining the amount of test compound, if any, which is bound to the protein. Such a method may involve the incubation of the test compound and the protein immobilized on a solid support. Still other conventional methods of drug screening can involve employing a suitable computer program to determine compounds having similar or complementary structure to that of the gene product or portions thereof and screening those compounds for competitive binding to the protein. Such compounds may be incorporated into an appropriate therapeutic formulation, alone or in combination with other active ingredients. Methods of formulating such therapeutic compositions, as well as suitable pharmaceutical carriers, and the like are well known to those of skill in the art.

Accordingly, through use of such methods, the present invention is believed to provide compounds capable of interacting with these genes, or encoded proteins or fragments thereof, and either enhancing or decreasing the biological activity, as desired. Such compounds are also encompassed by this invention.

Numerous modifications and variations of the present invention are included in the above-identified specification and are expected to be obvious to one of skill in the art. Such modification and alterations to the compositions and processes of the present invention are believed to be encompassed in the scope of the claims appended hereto.

Each reference disclosed herein is incorporated by reference herein in its entirety. Any patent application to which this application claims priority is also incorporated by reference herein in its entirety.

The following nonlimiting examples are provided to further illustrate the present invention.

EXAMPLES

Example 1: Gene Essentiality

The essentiality of genes expressed in *S. aureus* infections is determined by introduction of 10^2 -10^8 cells infected with a selected strain of *S. aureus*, for example, RN4220, into an appropriate animal model such as the murine wound or pyelonephritis models. Infected tissues are recovered from the animals at various times post infection. Total RNA is isolated from the tissues and subjected to RT-PCR directed by primers designed using a *S. aureus* genome database to amplify all transcripts encoded by the *S. aureus* strain under study. The promoters of genes whose transcripts are temporally and conditionally controlled are then ligated into an *E. coli*/*S. aureus* shuttle vector at the unique *Hin*dIII site of pCU1 in accordance with procedures described by Augustin et al., *Eur. J. Biochem.*, 1992, 204:1149-1154. This shuttle vector also carries the reporter gene, luc, containing appropriate codons for *S. aureus* translation inserted in the unique *Eco*R1 site of pCU1. Recombinants are amplified in *E. coli* using resistance to ampicillin (100 mg/ml) as a selection.

The recombinant plasmids so generated are then isolated from *E. coli* and introduced by electroporation into *S. aureus* RN4220, selecting for chloramphenicol resistance (10 mg/ml) and then transferred into the virulent *S. aureus* strain WCUH29 modified to carry the genes for the biosynthesis and recycling of the substrate and fatty acid for the luciferase reaction inserted in the chromosome at a position known to have no effect on virulence. Luciferase expression is determined visually by looking for luminescence in thin tissue sections or by RT-PCR analysis for luciferase specific

mRNA both *in vitro* and *in vivo* to confirm whether the characteristics of the promoter have been preserved. If the "on/off" characteristics of the promoter have been preserved, the coding sequence of the luciferase gene is replaced by that of a ribozyme or antisense RNA specific for a candidate gene under study. The new construct is inserted into *S. aureus* modified to carry the entire luciferase operon in the chromosome. Expression during *in vitro* growth of the ribozyme or antisense RNA as well as its target RNA is monitored to confirm appropriate expression. The expression of both is then be monitored during infection by growing the *S. aureus* carrying the constructs in the presence of antibiotic overnight and introducing 10^2-10^8 viable cell counts into the animal model. Reduction in target mRNA is correlated with infection progression including disease pathology, luminescence in thin tissue sections to allow determination of the numbers of metabolically active bacteria and viable cell counts so that gene candidates can be prioritized for development of therapeutic agents.

Example 2: *Staphylococcus aureus* promoter that is "on" in an *in vivo* model

A *S. aureus* promoter sequence is provided as an exemplifcation of a promoter that may be modulated in "on" and "off" modes of expression. This promoter sequence for the open reading frame (herein "ORF") is transcribed *in vivo* in animal models of infection, but not *in vitro*. This promoter may be used in the methods of the invention.

There are two preferred putative promoter sequence regions indicated with underlining. Sequences in SEQ ID NO: I (below) that are underscored and in bold indicates the most preferred putative promoter region. Upstream of this is a second preferred putative promoter region (underscored only). The ATG start codon of the gene transcribed from the sequence of SEQ ID NO: 1 is italicized. Genes to be tested in the methods described herein may be expressibly ligated to the promoter sequence including or excluding this start codon.

[SEQ ID NO:1]

5'-GATGCAGAAGCGATTTACACGTACGAAGGT

ACACATGAAATTAATGCCTTAGTAATTGGACGCGCTTTGACTGGAGATTCTGCTTTCGTATAAATAGC

AAATAATTTATATGAGATGCATTAATTTCACTAAAAAAGACTTATTTTAAGCATAAAGCTTTTTCCTTA

AATAAGAGGCTAAG**ATGACTGTCAAAGATACTTAATTAATTTT**ATAAAATAGCAACGTTATTCCAATT

ATCTTAATGGTTATCTTATCCTCAACTAAATTGGAGGAATCACT*ATG*. . . .3'

SEQUENCE LISTING

(1) GENERAL INFORMATION

(i) APPLICANT: SmithKline Beecham Corporation and SmithKline Beecham p.l.c.

(ii) TITLE OF THE INVENTION: METHOD FOR DETERMINING GENE ESSENTIALITY IN A PATHOGEN

(iii) NUMBER OF SEQUENCES: 1

(iv) CORRESPONDENCE ADDRESS:

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(D) STATE: Middlesex

(E) COUNTRY: GB

(F) POST CODE: TW8 9EP

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Diskette

(B) COMPUTER: IBM Compatible

(C) OPERATING SYSTEM: DOS

(D) SOFTWARE: FastSEQ for Windows Version 2.0

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:

(B) FILING DATE: 15-OCT-1997

(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:

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(C) TELEX:

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 281 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

GATGCAGAAG CGATTTACAC GTACGAAGGT ACACATGAAA TTAATGCCTT AGTAATTGGA 60

CGCGCTTTGA CTGGAGATTC TGCTTTCGTA TAAATAGCAA ATAATTATAT GAGATGCATT 120

AATTTCACTA AAAAAGACTT ATTTTAAGCA TAAAGCTTTT TCCTTAAATA AGAGGCTAAG 180

ATGACTGTCA AAGATACTTA ATTAATTTTA TAAAATAGCA ACGTTATTCC AATTATCTTA 240

ATGGTTATCT TATCCTCAAC TAAATTGGAG GAATCACTAT G 281

Claims

1. A method of determining gene essentiality in a selected pathogen comprising:

(a) preparing a vector expression system comprising a promoter of the potential target gene having on/off characteristics and a reporter gene;
(b) expressibly linking the target gene promoter in the vector expression system with a ribozyme or antisense construct for the potential target gene;
(c) introducing the expression vector system containing the ribozyme or antisense construct into a selected pathogen;
(d) infecting at least one animal with the pathogen containing the expression vector system;
(e) determining gene expression levels of the target gene; and
(f) correlating target gene expression levels with progression of the infection in the animal to identify genes essential to growth of the selected pathogen.

2. A method of determining gene essentiality in a selected pathogen comprising:

(a) preparing a vector expression system comprising a promoter of the potential target gene having on/off characteristics and a reporter gene;
(b) expressibly linking the target gene promoter in the vector expression system with a ribozyme or antisense construct for the potential target gene;
(c) introducing the expression vector system containing the ribozyme or antisense construct into a selected pathogen;
(d) infecting at least one animal with the pathogen containing the expression vector system;
(e) determining levels of mRNA of the target gene; and
(f) correlating levels of mRNA with progression of the infection in the animal to identify genes essential to growth of the selected pathogen.

3. A method of determining gene essentiality in a selected pathogen comprising:

(a) identifying a potential target gene;
(b) preparing a vector expression system comprising a promoter of the potential target gene having on/off characteristics and a reporter gene;
(c) confirming that the on/off characteristics of the promoter in the vector expression system are preserved;
(d) replacing the reporter gene in the vector expression system with a ribozyme or antisense construct for the potential target gene;
(e) introducing the expression vector system containing the ribozyme or antisense construct into a selected pathogen;
(f) infecting at least one animal with the pathogen containing the expression vector system;
(g) determining levels of mRNA of the target gene; and
(h) correlating levels of mRNA with progression of the infection in the animal to identify genes essential to growth of the selected pathogen.

4. A method of determining gene essentiality in a selected pathogen comprising:

(a) preparing a vector expression system comprising a promoter of the potential target gene having on/off characteristics and a reporter gene;
(b) expressibly linking the target gene promoter in the vector expression system with a ribozyme or antisense construct for the potential target gene;
(c) introducing the expression vector system containing the ribozyme or antisense construct into a selected pathogen;
(d) infecting at least one animal with the pathogen containing the expression vector system;
(e) determining levels of protein of expressed by the target gene; and
(f) correlating levels of protein with progression of the infection in the animal to identify genes and gene products essential to growth of the selected pathogen.

5. The method of claim 1 wherein the selected pathogen is selected from the group consisting of *Streptococcus, Streptococcus pneumoniae, Staphylococcus, Staphylococcus aureus, Enterococcus, Enterococcus faecalis, Pseudomonas, Pseudomonas auriginosa, Escherichia,* and *Escherichia coli.*

6. The method of claim 2 wherein the selected pathogen is selected from the group consisting of *Streptococcus, Streptococcus pneumoniae, Staphylococcus, Staphylococcus aureus, Enterococcus, Enterococcus faecalis,*

Pseudomonas, Pseudomonas auriginosa, Escherichia, and *Escherichia coli.*

7. The method of claim 3 wherein the selected pathogen is selected from the group consisting of *Streptococcus, Streptococcus pneumoniae, Staphylococcus, Staphylococcus aureus, Enterococcus, Enterococcus faecalis, Pseudomonas, Pseudomonas auriginosa, Escherichia,* and *Escherichia coli.*

8. The method of claim 4 wherein the selected pathogen is selected from the group consisting of *Streptococcus, Streptococcus pneumoniae, Staphylococcus, Staphylococcus aureus, Enterococcus, Enterococcus faecalis, Pseudomonas, Pseudomonas auriginosa, Escherichia,* and *Escherichia coli.*

9. The method of claim 1 wherein the animal of the infecting step is selected from the group consisting of a rodent, a mouse, a rat, a rabbit, and a guinea pig.

10. The method of claim 2 wherein the animal of the infecting step is selected from the group consisting of a rodent, a mouse, a rat, a rabbit, and a guinea pig.

11. The method of claim 3 wherein the animal of the infecting step is selected from the group consisting of a rodent, a mouse, a rat, a rabbit, and a guinea pig.

12. The method of claim 4 wherein the animal of the infecting step is selected from the group consisting of a rodent, a mouse, a rat, a rabbit, and a guinea pig.

13. A kit comprising the vector of claim 1.

14. The vector of claim 1.

15. The pathogen of claim 1.

16. An expression vector comprising a promoter of a potential target gene having on/off characteristics and a reporter gene.

17. A vector comprising a target gene promoter expressibly linked to a ribozyme or antisense construct for the potential target gene.

18. A pathogen comprising an expression vector comprising a promoter of a potential target gene having on/off characteristics and a reporter gene.

19. A pathogen comprising a vector comprising a target gene promoter expressibly linked to a ribozyme or antisense construct for the potential target gene.

20. An animal comprising the pathogen of claim 19.

European Patent Office

EUROPEAN SEARCH REPORT

Application Number
EP 97 30 8157

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.6)
A	EP 0 467 349 A (UNIV NEW YORK) * the whole document * ---	1	C12Q1/68 C12N15/63 C12N15/31
D,A	HENSEL M ET AL: "SIMULTANEOUS IDENTIFICATION OF BACTERIAL VIRULENCE GENES BY NEGATIVE SELECTION" SCIENCE, vol. 269, 21 July 1995, pages 400-403, XP000645478 * the whole document * ---	1	C12N15/74 C12N1/21 C12N15/00
A	GUERRIER-TAKADA C ET AL: "Atrificial regulation of gene expression in Escherichia coli by RNase P" PROC. NATL. ACAD. SCI. USA, vol. 92, November 1995, pages 11115-9, XP002052832 * the whole document * ---	1	
A	WO 95 29245 A (ASS POUR LE DEV DE LA RECH ;EGLY JEAN MARC (FR); HUMBERT SANDRINE) * the whole document * ---	1	TECHNICAL FIELDS SEARCHED (Int.Cl.6) C12Q
A	WO 95 30755 A (HISAMITSU PHARMACEUTICAL CO ;SHIMADA TAKASHI (JP); AKIYAMA KATSUHI) see abstract ---	1	
A	EP 0 628 635 A (NUNHEMS ZADEN BV) * the whole document * ---	1	
A	WO 92 01806 A (PUBLIC HEALTH RESEARCH INST OF) * claims 1-13,37-47 * -----	1	

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	26 January 1998	Osborne, H

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
O : non-written disclosure
P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

EPO FORM 1503 03.82 (P04C01)

JOURNAL OF BACTERIOLOGY, Nov. 1999, p. 6585–6590
0021-9193/99/$04.00+0

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Regulated Antisense RNA Eliminates Alpha-Toxin Virulence in *Staphylococcus aureus* Infection

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The ability to selectively disrupt gene function remains a critical element in elucidating information regarding gene essentiality for bacterial growth and/or pathogenesis. In this study, we adapted a *tet* regulatory expression system for use in *Staphylococcus aureus*, with the goal of downregulating gene expression via induction of antisense RNA. We demonstrate that this system exhibits a 50- to 100-fold dose-dependent level of induction in bacterial cells grown in culture (i.e., in vitro) and also functions in mice (i.e., in vivo) following oral administration of inducer. To determine whether induced antisense RNA could interfere with chromosomally derived gene expression, we cloned a fragment of the *S. aureus* alpha-toxin gene (*hla*) in antisense orientation downstream of the *tet* promoter system and introduced the construct into *S. aureus*. Induced antisense *hla* RNA downregulated chromosomally derived *hla* gene expression in vitro approximately 14-fold. Similarly, induction of *hla* antisense RNA in vivo dramatically reduced alpha-toxin expression in two different murine models of *S. aureus* infection. Most importantly, this reduction completely eliminated the lethality of the infection. These results indicate that the *tet* regulatory system functions efficiently in *S. aureus* and induced antisense RNA can effectively downregulate chromosomal gene expression both in vitro and in vivo.

Selective disruption and/or downregulation of gene expression is an important tool for elucidating information on gene essentiality for bacterial growth or pathogenesis. This information is particularly useful for validating appropriate molecular targets for antibiotic discovery. A variety of techniques have been developed in various bacterial systems to achieve functional inactivation of gene products (4, 5, 10, 13). Most of these involve gene knockout methods using point mutation, insertional inactivation, or deletion (e.g. allelic replacement).

A possible refinement over gene knockout technology has been the introduction of controlled gene expression systems that allow particular genes to be regulated and thereby functionally analyzed (7, 23). Information is gained by switching off or on gene expression and monitoring the effect as the product titrates down or up. These systems have the potential to provide more quantitative data on the functional importance of a gene product to either growth or virulence. These regulated systems are best described for *Escherichia coli* and, more recently, *Bacillus subtilis* (7). Moreover, they are now beginning to be developed for other pathogens (23).

Another effective means of reducing gene expression has been the use of antisense technology. Antisense methods have been used effectively to downregulate eukaryotic gene expression in a variety of systems (1, 3, 6, 19). These methods have not been routinely adapted for prokaryotes despite the fact that antisense regulation has been shown to be a natural phenomenon in bacteria (25). This may be because of the other options available for bacterial systems. However, recent reports have demonstrated that antisense approaches using various synthetic oligomers can be used effectively in bacteria (8, 14, 22, 26).

The gram-positive bacterium *Staphylococcus aureus* represents a serious human health threat, causing such varied infections as skin abscesses, osteomyelitis, endocarditis, septicemia, and pneumonia. There exist a variety of genetic tools and approaches for generating and analyzing null mutants in *S. aureus* (4, 24). However, a facile system for selectively controlling and downregulating specific staphylococcal gene products for analysis both in vitro and in vivo is lacking. The ability to titrate down a gene product either under culture conditions or in an animal model of infection would provide a powerful, additional approach for studying gene essentiality and pathogenesis in this organism.

In this study, we adapted a regulated expression system for use in *S. aureus*. We show its utility in regulating the expression of an antisense construct of the staphylococcal alpha-toxin gene (*hla*) and, in turn, regulating the production of the alpha-toxin itself. Most importantly, we demonstrate that induced expression of antisense *hla* RNA is achieved both in in vitro culture and in two murine models of staphylococcal infection, and downregulation of the toxin reverses its lethal phenotype in an intraperitoneal infection.

MATERIALS AND METHODS

Bacterial strains and plasmids. The plasmids used in this study were pUC19 (27), pE194 (11), pMH109 (12), and pWH353 (kindly provided by W. Hillen, Institut für Mikrobiologie und Biochemie, Erlangen, Germany). The bacterial strains used in this study were *S. aureus* RN4220, a derivative of *S. aureus* 8325-4 that is able to accept transformed DNA (18), and *S. aureus* WCUH29, a virulent alpha-toxin-producing clinical isolate. *S. aureus* strains were cultured in tryptic soy broth (TSB; BBL, Sparks, Md.) or TSB plus 1.5% Bacto Agar (TSA; Difco, Detroit, Mich.). To maintain selection of plasmid pYJ90, *S. aureus* was grown in culture medium containing erythromycin (Erm; 5 μg/ml). *Escherichia coli* DH5-α, used for construction of shuttle vectors, was grown in Luria-Bertani (LB) broth (BBL) containing chloramphenicol (Cam; 20 μg/ml), Erm (300 μg/ml), or ampicillin (Ap; 100 μg/ml) as appropriate.

Construction of *E. coli-S. aureus* shuttle vector pYJ90. To construct a suitable shuttle vector for this study, plasmids pUC19 carrying an Ap^r marker (27) and pE194 carrying an Erm^r marker (11) were each digested with *Nde*I, gel purified, ligated together, and transformed into *E. coli* DH5-α by electroporation. Transformants were selected on LB agar containing Ap (100 μg/ml) and Erm (300 μg/ml). Transformants were examined by restriction analysis to identify an appropriate shuttle plasmid containing both pUC19 and pE194 fragments. One recombinant, pYJ90, was confirmed by further restriction enzyme digestion. pYJ90 was then electroporated into *S. aureus* RN4220 (15, 18). Transformants

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were selected on TSA containing Erm (5 μg/ml). The stability of plasmid pYJ90 in *S. aureus* was determined by passaging a culture six times in medium with antibiotics and analyzing plasmid DNA in the bacterial culture.

Construction of a *tet* regulatory system in plasmid pYJ90. The *Cla*I-*Hin*dIII fragment containing the *tetR* gene (which encodes the *tet* repressor), its promoter (P_R), and the strong *xyl/tet* promoter-operator fusion ($P_{xyl/tetO}$) was excised from plasmid pWH353 (7) and cloned between the *Cla*I and *Hin*dIII sites in plasmid pBluescript II KS (Stratagene, La Jolla, Calif.). The resulting plasmid, pYJ101, was digested with *Eco*RI and *Pst*I and an *Eco*RI-*Pst*I fragment containing a promoterless *cat* (chloramphenicol acetyltransferase [CAT]-encoding) gene followed by a transcriptional terminator (derived from pMH109 (12), and adapted with the appropriate *Eco*RI and *Pst*I restriction sites by first moving it into the *Eco*RI and *Bam*HI sites of pUC19), was inserted. This new construct was named pYJ103. pYJ103 was digested with *Sal*I, and the fragment containing the *tetR*/P_R/$P_{xyl/tetO}$-*cat* region was gel purified and cloned into the *Sal*I site pYJ90. The resulting plasmid, pYJ335, was confirmed by restriction enzyme digestion and DNA sequencing and then electroporated into *S. aureus* RN4220. One of the transformants, YJSB335, was confirmed and used to make phage lysates by using *S. aureus* phage φ11 (2).

Construction of plasmid pYJ335 containing antisense *hla* and sense *hla*. A 621-bp *hla* fragment carrying 354 bp of N-terminal coding region of the *hla* gene and 268 bp 5′ to this region was generated by PCR amplification using primers *hla*For64 (5′ GGGGGGCCCGGGTATGTCTTTTCCTTGTTTCA 3′) and *hla*Rev684 (5′GGGGGGCCCGGGATCAGGTAGTTGCAACTG 3′), corresponding to nucleotides 64 to 83 and 684 to 701, respectively (9). Boldface nucleotides correspond to the *Sma*I restriction enzyme recognition site, and underlined nucleotides correspond to the *hla* coding sequence. The amplified *hla* fragment contains the *hla* promoter region. The PCR product was digested with *Sma*I, gel purified, and ligated into the *Eco*RV site of downstream of the *xyl/tetO* promoter-operator fusion of pYJ335. The orientation of the *hla* insertion was determined by PCR. As expected, only recombinants containing the *hla* fragment in the antisense orientation yielded a PCR product of approximately 800 bp, using primers *tetR*For1399 and *hla*For64. In contrast, only recombinants containing the *hla* fragment in the sense orientation produced a PCR product of approximately 800 bp, using primers *tetR*For1399 and *hla*Rev684 (data not shown). The resulting plasmids, pYJ318-7 and pYJ318-16, which contained *hla* in the antisense and sense orientations, respectively, were electroporated separately into *S. aureus*. Transformants were selected by Erm resistance, and plasmids were confirmed by restriction enzyme digestion. Transformants YJSB318-7 and YJSB318-16 containing antisense *hla* and sense *hla*, respectively, were confirmed and used to make φ11 phage lysates.

***S. aureus* transductions.** The clinical isolate WCUH29 cannot be transformed directly by electroporation. Therefore, plasmids pYJ335, pYJ318-7, and pJY318-16 were introduced into this strain by phage transduction (2). Phage φ11 was used to make phage lysates by infecting *S. aureus* YJSB335, YJSB318-7, and YJSB318-16 grown in top agar (TSB containing 0.7% agar and 5 mM $CaCl_2$). The phage lysates were sterilized by passing each through a 0.45-μm-pore-size filter and titered on *S. aureus* RN4220. Transductions were performed by incubating 5×10^9 CFU of WCUH29 cells with 100 μl of phage lysate (10^9 to 10^{10} PFU) and 5 mM $CaCl_2$ at 37°C for 30 min. One milliliter of ice-cold 20 mM sodium citrate was then added to the above mixture to block phage adsorption. The bacterial cells were centrifuged and resuspended in 500 μl of 20 mM sodium citrate. Transductants were selected on TSB-agar containing sodium citrate (500 μg/ml) and Erm (5 μg/ml), and transductants YJ335, YJ318-7, and YJ318-16, containing plasmids pYJ335, pYJ318-7, and pYJ318-16, respectively, were confirmed by restriction enzyme digestion.

PCR, RT-PCR, and DNA sequencing techniques. The 621-bp *hla* fragment was generated by PCR using *hla*-specific primers. The antisense *hla* and sense *hla* orientations in plasmids pYJ318-7 and pYJ318-16, respectively, were confirmed by PCR using the plasmid-specific primer *tetR*For1399 (5′ CAATACATTGTA GGCTGC 3′), corresponding to nucleotides 1399 to 1416 and *hla*-specific primers *hla*Rev684 and *hla*For64. The reaction conditions for all PCRs were 0.2 mM deoxynucleoside triphosphates, 2.5 mM $MgCl_2$, 50 pmol of each primer, 1 ng of template DNA, and 2.5 U of *Taq* polymerase in buffer supplied by the manufacturer (Gibco-BRL, Rockville, Md.). For the antisense *hla* and sense *hla* orientations, the primers were *tetR*For1399 plus *hla*For64 and *tetR*For1399 plus *hla*Rev684, respectively, using the same annealing temperature of 48°C. For reverse transcription (RT)-PCR analysis, bacterial RNA was isolated from infected tissue samples by using FastRNA reagents (Bio 101, Vista, Calif.) and treated with RNase-free DNase I (GeneHunter Corporation, Nashville, Tenn.) to remove DNA. Single-stranded cDNA was synthesized by incubating DNase-treated RNA with reverse transcriptase in reaction buffer containing random hexamer primers supplied by the manufacturer (Gibco-BRL). After RNase H treatment, cDNA was used as the template for PCR using the *tetR-cat*-specific primers *tetR*For1399 and *cat*Rev768 (5′ GGCAGGTTAGTGACATTAG 3′) and the *hla* gene-specific primers *hla*For64 and *hla*Rev684. DNA sequencing was performed to further confirm the *tet* regulatory elements in pYJ335 and the antisense *hla* and sense *hla* orientations in pYJ318-7 and pYJ318-16, respectively.

Specific CAT activity assays. CAT activity was determined spectrophotometrically as described by Shaw (20), using kinetic SoFTmax PRO II software (Molecular Devices Corporation, Sunnyvale, Calif.) to monitor activity. Briefly, *S. aureus* YJ335 was grown with shaking in TSB-Erm at 37°C to an A_{600} of 0.25. The culture was divided, and different doses (0, 2.5, 25, 250, 500, and 1,000 ng/ml) of tetracycline (Tc) were added to the cultures. Two milliliters was removed from each culture 3 h after the addition of Tc for the dose-dependent assay or after 0, 1, 2, 3, and 4 h following the addition of Tc for the time course assay. The bacterial cells were harvested by centrifugation and washed once with 25 mM Tris (pH 7.8)–10 mM EDTA buffer. Crude protein extracts were prepared by centrifugation after the bacterial cells had been suspended in 200 μl of the same buffer containing lysostaphin (0.2 mg/ml; Sigma, St. Louis, Mo.) and incubated at 37°C for 10 min. The total protein concentration was determined by using the Bio-Rad protein microassay (Bio-Rad Laboratories, Hercules, Calif.). Specific CAT activity was calculated as the number of units of CAT activity per milligram of total protein. Experiments were performed in triplicate at least twice, and similar results were obtained.

Northern blot analysis. *S. aureus* YJ318-7 and YJ318-16 were grown in TSB-Erm to an A_{600} of 0.25 with and without Tc (250 ng/ml), and total RNA was extracted by using a Qiagen RNeasy mini protocol kit (Qiagen, Incorporated, Chatsworth, Calif.). Ten-microgram aliquots of total RNA from YJ318-7 and YJ318-16 grown in the presence and absence of Tc (250 ng/ml) were separated by electrophoresis on a 1.2% agarose–1.8% formaldehyde gel and blotted onto a nylon membrane (Boehringer Mannheim Biochemicals, Indianapolis, Ind.). RNA was cross-linked to the membrane by UV irradiation by using a UV Stratalinker (Stratagene). Blots were prehybridized and then hybridized with digoxigenin (DIG)-labeled single-stranded DNA oligonucleotides in high-sodium dodecyl sulfate (SDS) buffer (Boehringer Mannheim Biochemicals) at 50°C for 6 h. Single-stranded DNA oligonucleotides specific hybridization with either sense *hla* RNA (5′ GGCCAGGCTAAACCACTTTTGTTAGCACCTTCTTCG CTATAAACTCTATA 3′) or antisense *hla* RNA (5′ TATAGAGTTTATAGC GAAGAAGGTGCTAACAAAAGTGGTTTAGCCTGGCC 3′) were labeled by 3′ tailing DIG-dUTP (Boehringer Mannheim Biochemicals), and 100 pmol of each was used to probe the membranes. The DIG-DNA-RNA hybridization on a nylon membrane was detected with DIG luminescent detection reagents (Boehringer Mannheim Biochemicals) and exposed to X-ray film.

Western blot analysis. For preparation of extracellular protein, Tc was added to 10-ml cultures of *S. aureus* WCUH29, YJ318-7, and YJ318-16 to a final concentration of 250 ng/ml and incubated with shaking at 37°C for 8 h. Supernatants were collected after centrifugation, transferred into tubes containing an equal volume of ethanol, and incubated overnight at 4°C. Extracellular proteins were precipitated by centrifugation at 15,000 × *g* at 4°C for 30 min. SDS-polyacrylamide gel electrophoresis (PAGE) and Western blotting methods were performed as previously described (16). Equal amounts of protein were loaded into each lane of an SDS–12.5% polyacrylamide gel. The primary antiserum used to detect alpha-toxin was provided by M. Burnham, SmithKline Beecham Pharmaceuticals. Standard alpha-toxin and anti-rabbit antibody-alkaline phosphatase conjugate were from Sigma. Western blots were scanned by using Eagle Eye-II software (Stratagene) to quantitate protein bands.

Murine hematogenous pyelonephritis and intraperitoneal infection models. CD-1 female mice (25 g) obtained from Charles River Laboratories were used for in vivo testing. *S. aureus* WCUH29, YJ335, YJ318-7, and YJ318-16 were harvested from 1 ml of stationary-phase culture, washed once with 1 ml of phosphate-buffered saline (PBS), and diluted to an A_{600} of 0.2. These bacterial suspensions were diluted and plated onto TSB-agar plates for determination of viable CFU. A total of six mice per group were infected with approximately 10^7 CFU of bacteria via an intravenous injection of 0.2 ml of bacterial suspension into the tail vein via a tuberculin syringe. Sublethal doses of Tc (0.5 μg/g) and Erm (5 ng/g) to maintain plasmids were given orally (0.1 ml/mouse) to infected mice on days 1, 2, and 3 after infection. The mice were sacrificed by carbon dioxide overdose 2 h after the last dose of Tc, and kidneys were aseptically removed. The kidneys from three mice in each group were embedded in O.C.T. compound (Tissue-Tek, Torrance, Calif.), frozen in liquid nitrogen, and kept at −80°C for immunostaining. The kidneys from the remaining three mice were cut in half; one half was snap-frozen in cryovials in liquid nitrogen for RT-PCR analysis, and the other half was homogenized in 1 ml of PBS for enumeration of viable bacteria.

For murine intraperitoneal infection, female CD1 mice (10 in each experimental group) were infected as previously described (14). Briefly, 18-h cultures of *S. aureus* (about 3×10^9 CFU/ml) were collected by centrifugation at 3,000 × *g* for 10 min, washed three times with PBS, and resuspended in PBS, and 0.5-ml aliquots of the bacterial solutions were injected intraperitoneally. Viable counts were determined by plating dilutions of cultures on TSB-agar Erm plates.

Immunohistochemistry. Sections of 8 μm were cut from the frozen embedded kidney tissue in O.C.T. compound, mounted onto microslides (selected, precleaned Superfrost Plus; VWR Scientific, West Chester, Pa.), and fixed with cold methanol. The fixed tissue sections were washed in PBS and incubated in 5% dry milk–PBS. The tissue sections were then incubated with specific rabbit anti-alpha-toxin serum at room temperature for 1 h, washed with 0.1% goat serum–PBS, and incubated with biotinylated donkey anti-rabbit antibodies (1:200; Amersham Life Science, Piscataway, N.J.) for 1 h at room temperature. The tissue sections were washed in PBS, processed with Vectastain ABC reagents (Vector Laboratories, Burlingame, Calif.), and washed in PBS again, after which the peroxidase substrate 3,3′-diaminobenzidine (Sigma Fast; Sigma) was added. The sections were washed with tap water, dehydrated, and mounted with coverslips.

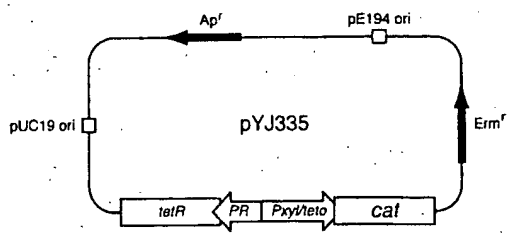

FIG. 1. Tc-inducible shuttle vector pYJ335 (see Materials and Methods for details). pUC19 ori and pE194 ori, origins of replication from pUC19 and pE194, respectively, allowing plasmid replication in *E. coli* and *S. aureus* hosts. A unique *Eco*RV site is positioned downstream from the *xyl/tet* promoter and 22 nucleotides upstream of the translation start point of the *cat* gene.

RESULTS

Adaptation of the *tet* regulatory system to *S. aureus*. To assess both the function and regulation of the *xyl/tet* promoter-operator control system, we fused it to a *cat* gene reporter construct on an *E. coli-S. aureus* plasmid shuttle vector (Fig. 1) and transferred it into *S. aureus* (See Materials and Methods for details). The resulting transformant, YJ335, was used to examine the expression of CAT activity in response to Tc induction. The results are shown in Fig. 2A. In the absence of Tc, YJ335 showed low basal levels of CAT activity. In contrast, upon addition of Tc to the culture medium, CAT expression was induced efficiently in a dose-dependent manner, with maximal activity seen 3 h after induction with 250 ng of Tc per ml. Higher Tc doses resulted in decreased levels of CAT expression, presumably due to the effects of the antibiotic on bacterial growth. Our results demonstrate that a strong, dose-dependent induction response can be achieved with this system in *S. aureus*.

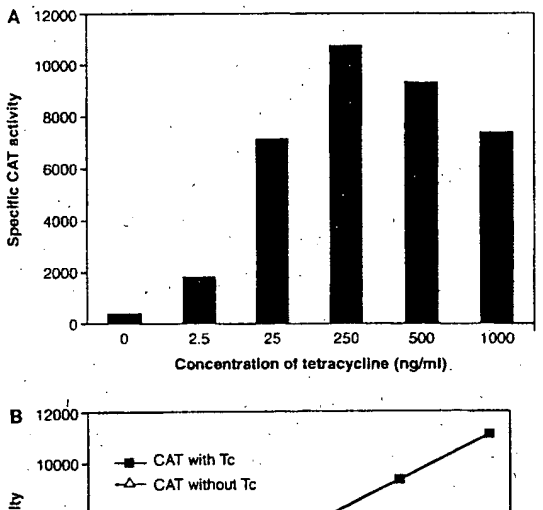

FIG. 2. Dependence of CAT activity on Tc concentration (A) and kinetics of Tc induction in *S. aureus* (B). (A) *S. aureus* YJ335 was incubated in TSB with 5 ng of Erm per ml to early log phase, and different doses of Tc were added to aliquoted cultures; 2 ml of each culture was transferred into a new tube, and the cells were harvested by centrifugation 3 h after the addition of Tc. (B) Strain YJ335 was incubated to early log phase in TSB, and 250 ng of Tc per ml was added to the culture; 2-ml aliquots of culture were collected 0, 1, 2, 3, and 4 h after addition of Tc. Crude protein preparations were used to analyze CAT activity. Specific CAT activity is defined as units of CAT activity per milligram of total protein.

We also examined the time dependence of CAT expression at 250 ng of Tc per ml. The results (Fig. 2B) indicate that in the absence of Tc, low basal expression was maintained over a 3- to 4-h period. In the presence of Tc, CAT activity increased steadily and achieved a 50- to 100-fold induction between 1 and 4 h. Apparently the *tet* regulatory system can be used effectively in *S. aureus* to regulate expression of a gene placed under its control.

Induced antisense RNA downregulates gene expression in vitro. Next, we wanted to use this plasmid based *tet* regulation system in *S. aureus* to induce a specific antisense transcript and measure its effect on the expression of the cognate gene located on the staphylococcal chromosome. We chose for this analysis a 621-bp DNA fragment containing a portion of the staphylococcal alpha-toxin gene (*hla*). This gene was chosen because it is a known virulence factor (2, 14) and reagents were readily available for monitoring its expression. The *hla* fragment was inserted in each of its orientations (sense and antisense) downstream from the *tet*-regulated promoter (Fig. 1). The two plasmids were introduced separately into *S. aureus*, thereby creating the isogenic transformants YJ318-7 (antisense) and YJ318-16 (sense). These strains were used to characterize the production of the *hla* fragment transcript in response to Tc and to examine the effects of this induction on alpha-toxin gene expression driven from the normal *hla* chromosomal locus.

First, we examined, by Northern blot analysis, RNA production from both the sense (YJ318-16) and antisense (YJ318-7) constructs before and after induction. The results (Fig. 3A) show that YJ318-7 selectively produced a major antisense *hla* RNA product in response to Tc induction (lane 5). Antisense RNA was not detected in the absence of induction (lane 6), nor was any sense RNA produced with or without Tc (lanes 1 and 2). Correspondingly, the control sense construct, YJ318-16, produced sense *hla* fragment transcript only in response to Tc (lane 3). Again, it appears that the plasmid-based regulation system operated effectively in *S. aureus*.

Second, we examined the effect of these induced *hla* RNA segments on the expression of endogenous *hla* gene product. The culture supernatant of YJ318-7 without Tc induction reacted strongly with anti-alpha-toxin antisera (Fig. 3B), while YJ318-7, following Tc induction, demonstrated a 14-fold-lower reactivity as measured by densitometer scanning. In addition, the amount of alpha-toxin in the culture supernatant of YJ318-16, with or without induction, was similar to that of wild-type WCUH29. These results demonstrate the ability of this *tet* regulatory system to efficiently inhibit gene expression in *S. aureus*.

***tet*-regulated transcription during infection.** We used a standard murine renal infection model (21) to examine Tc-induced transcription from the *tet* regulatory system in vivo. *S. aureus* YJ335 carrying CAT expression vector pYJ335 (Fig. 1) was injected (10^7 CFU) into the mouse tail vein (see Materials and Methods for details). One day after infection, mice were either not treated or given Tc orally once a day for 3 days. On day 3, the kidneys were removed, and the total RNA was prepared and analyzed by RT-PCR using CAT gene-specific primers.

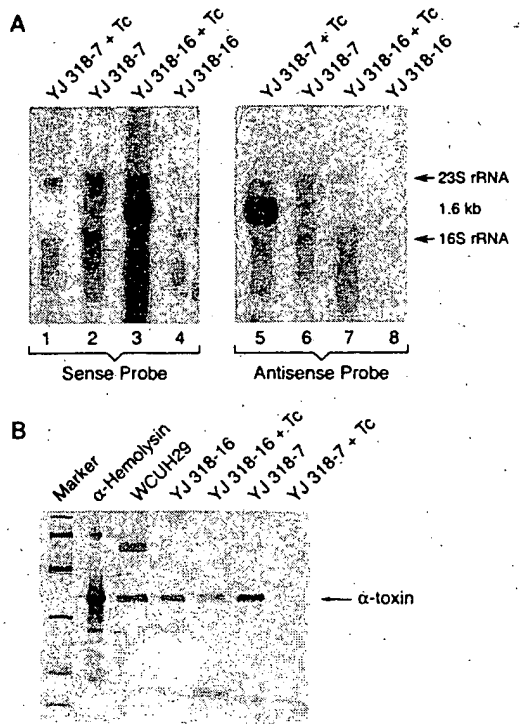

FIG. 3. Northern blot analysis of sense *hla* and antisense *hla* transcription (A). DIG-labeled single-stranded DNA oligonucleotide probes hybridized specifically with either sense *hla* RNA (sense probe) or antisense *hla* RNA (antisense probe). Hybridization of the 1.6-kb RNA represents the *hla-cat* cotranscripts. Western blot analysis of alpha-toxin expressed in strain WCUH29 and its isogenic strains with or without Tc induction (B). Lanes: 1, molecular weight markers (biotinylated low-molecular-weight SDS-PAGE standards; Bio-Rad); 2, alpha-hemolysin (Sigma) as positive standard control; 3, proteins from WCUH29 culture supernatant; 4, proteins from YJ318-16 culture supernatant without induction; 5, proteins from YJ318-16 culture supernatant with Tc induction; 6, proteins from YJ318-7 culture supernatant without induction; 7, proteins from YJ318-7 culture supernatant with Tc induction.

The results (shown in Fig. 4A) demonstrate the selective expression of CAT RNA in the infected mouse kidney (lane 3) in response to Tc induction. No PCR signal was detected in the absence of the RT reaction (lane 4), which demonstrates the lack of any plasmid DNA contamination in the RNA sample. Moreover, no signal was detected in the absence of Tc induction (lanes 1 and 2), thereby confirming that Tc-induced expression of CAT transcript was achieved in vivo.

We repeated this experiment in the same infection model using *S. aureus* YJ318-7 carrying the antisense construct of *hla*. The results (Fig. 4B) again demonstrate selective RT-PCR detection of the antisense *hla* transcript in the infected mouse kidney only in response to Tc induction (lanes 3 and 5).

Induced antisense RNA downregulates gene expression in vivo. To examine directly the consequences of antisense *hla* RNA induction in the murine kidney infection model, we monitored the expression of alpha-toxin in infected kidney tissue by using immunohistochemical staining (Fig. 5). As a control, we used wild-type *S. aureus* carrying the sense fragment *hla* expression vector, YJ318-16. Kidneys from mice infected with YJ318-16 in the presence and in the absence of Tc induction (Fig. 5C and D, respectively) exhibited strong *hla* staining, essentially identical to a wild-type staphylococcal infection without plasmid (data not shown). Similarly, kidneys infected with *S. aureus* carrying antisense *hla* construct YJ318-7, in the absence of induction, also showed very strong *hla* staining (Fig. 5A). In contrast, kidneys infected with YJ318-7 following induction of antisense *hla* RNA showed little to no staining (Fig. 5B). These results clearly indicate that *tet* regulation of the plasmid vector system functions effectively in vivo and significantly downregulates chromosomally derived *hla* gene expression.

To confirm and extend these results, we examined the effect of antisense *hla* RNA induction in a second standard mouse model of acute infection. In this case, the mice are infected by the intraperitoneal route (14), which results in death within 12 to 24 h. The results (Fig. 6) indicate that all animals infected with the control carrying the *hla* sense fragment construct, YJ318-16, died within 24 h of inoculation whether or not they were given the Tc inducer. Similarly, all mice infected with *S. aureus* carrying the antisense construct, YJ318-7, in the absence of Tc inducer died. In marked contrast, all 10 mice infected with YJ318-7 and subject to oral Tc induction survived throughout the 6-day experiment. Clearly, Tc induction of antisense *hla* RNA dramatically attenuates the virulence and lethal phenotype of the *S. aureus* infection.

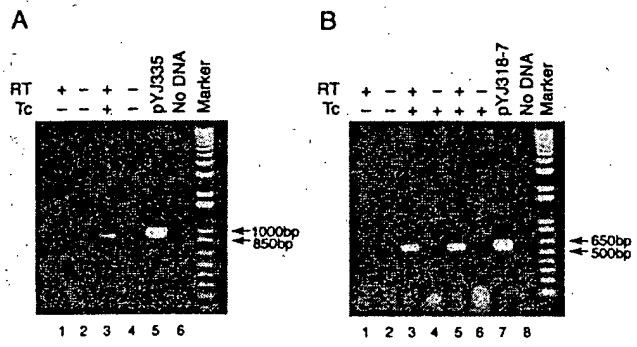

FIG. 4. RT-PCR analysis of transcription of *cat* (A, lane 3) and antisense *hla* (B, lanes 3 and 5) following in vivo induction with sublethal doses of Tc. The expected sizes of the RT-PCR products are 1 kb for *cat* and 620 bp for *hla*. Plasmid DNA template was used as a positive control for the expected PCR product (pYJ335 and pYJ318-7). Negative controls were samples prepared without RT or template DNA. Marker is the 1-Kb Plus DNA ladder from Gibco-BRL.

DISCUSSION

We have adapted the *tet*-regulated gene expression system originally developed for controlling expression of heterologous genes in *B. subtilis* (7) for use in a pathogenic strain of *S. aureus*. The results demonstrate that the *xyl/tet* hybrid promoter is functional in *S. aureus* and can increase expression of a reporter gene (i.e., *cat*) 50- to 100-fold. The extent of induction is similar to that seen in *B. subtilis* (7) and, more recently, in *S. pneumoniae* with an optimized plasmid-based promoter (23). Moreover, we show that in *S. aureus*, the induction is titrated in a dose-dependent manner, thereby allowing the system to be used for evaluating the level of expression required to achieve gene function and bacterial viability. However, we point out that the plasmid-based system we describe does exhibit some basal expression (albeit low [Fig. 2]) in the absence of induction, and this must be considered when interpreting any titration data obtained.

Because of the strong transcriptional induction achieved with the system, we examined its utility for producing antisense RNA in an efficient, regulated manner in order to selectively downregulate expression of specific chromosomal loci. We used an antisense fragment of the staphylococcal alpha-toxin

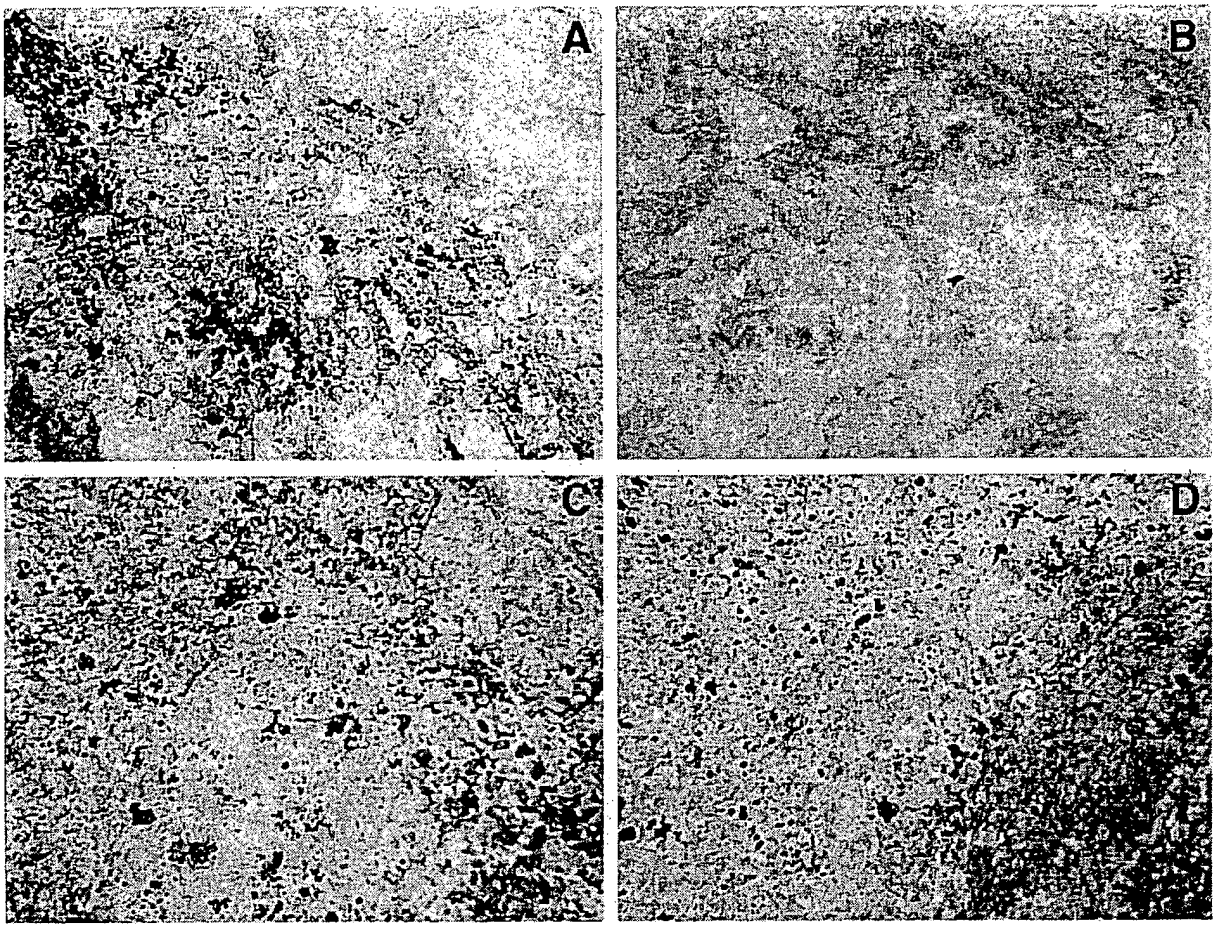

FIG. 5. Immunohistochemical detection of expression of alpha-toxin after in vivo induction of antisense *hla* RNA with sublethal doses of Tc. Sections of the kidneys were from mice infected with YJ318-7 in the absence (A) and presence (B) of Tc induction. Sections of the kidneys were from mice infected with YJ318-16 in the absence (C) and presence (D) of Tc induction. Magnification, ×400.

gene and demonstrated that the plasmid-based *tet* regulatory system can effectively produce an antisense transcript in response to induction. Importantly, this induction of antisense RNA was shown to effectively inhibit (~14-fold) *hla* expression in vitro. Thus, antisense regulation could be achieved in *S. aureus* and, coupled with a regulated promoter, provides a valuable tool for studying the effects of disrupting gene function.

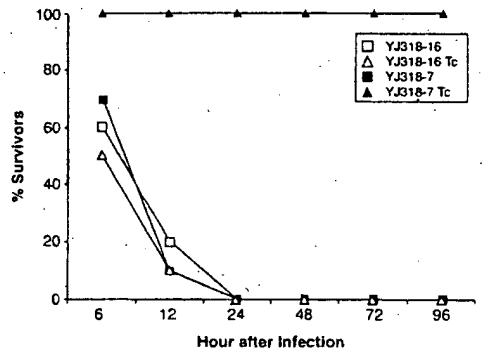

FIG. 6. Survival of mice infected intraperitoneally with strains YJ318-7 (1.4×10^9 CFU) and YJ318-16 (1.5×10^6 CFU) followed by oral induction with sublethal doses of Tc (YJ318-7 Tc and YJ318-16 Tc) or in the absence of Tc induction (YJ318-7 and YJ318-16).

We were particularly interested in extending the utility of our system to the in vivo analyses of staphylococcal gene function. The ability to examine the effects of downregulating specific bacterial genes in various animal infection models provides tremendous new scope for studying genes that may be essential for growth or virulence during infection. Our data for the murine pyelonephritis model demonstrate that *hla* expression (i.e., the production of staphylococcal alpha-toxin) can be regulated effectively via antisense RNA production. Direct examination of the infected mouse kidney shows a dramatic reduction in bacterial foci producing alpha-toxin. The few stained foci remaining (Fig. 5B) probably result from plasmid loss during the course of the infection study.

In the murine acute intraperitoneal infection model, we demonstrated that induction of antisense *hla* RNA abolished the lethal phenotype caused by expression of the toxin. Others have shown alpha-toxin to be a virulence factor in experimental infections such as peritonitis (17) and endocarditis (2). Our data support the importance of this gene product as a pathogene and provide a clear demonstration of the utility of regulated antisense production in vivo for examining virulence factors. By varying the time at which the antisense RNA is

induced (i.e., varying the time of oral Tc administration), this methodology should allow examination of the temporal relevance of specific gene products to the early establishment, maintenance, and postsymptomatic periods of the infection process. Thus, the system described should enable a detailed examination of the function of any staphylococcal gene throughout the infection process.

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